J Antimicrob Chemother
doi:10.1093/jac/dkx168

Journal of Antimicrobial Chemotherapy

Isolation of azole-resistant *Aspergillus fumigatus* from the environment in the south-eastern USA

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Received 8 December 2016; returned 14 February 2017; revised 17 March 2017; accepted 2 May 2017

Background: Azole resistance in isolates of the fungus *Aspergillus fumigatus* has been associated with agricultural use of azole fungicides. Environmental isolation of resistant isolates has been reported in Asia, Africa, Europe and South America.

Objectives: To determine whether *A. fumigatus* isolates containing TR_{34}/L98H or TR_{46}/Y121F/T289A can be found in fields in the USA treated with agricultural azoles.

Methods: Crop debris was collected and screened for *A. fumigatus*. All *A. fumigatus* isolates were screened for azole resistance. The *CYP51A* gene of azole-resistant isolates was sequenced. The population structure of a subset of isolates was determined using microsatellite typing.

Results: This article identifies azole-resistant *A. fumigatus* isolates containing the TR_{34}/L98H mutation in an experimental peanut field that had been treated with azole fungicides.

Conclusions: These findings suggest the development of resistance to azole antifungals in *A. fumigatus* may be present where agricultural azoles are used in the USA.

Introduction

Aspergillus fumigatus is a ubiquitous soil fungus found in decaying organic matter and can cause invasive infections in people with compromised immune systems such as those receiving immunosuppressive medication or transplant recipients.[1] The azole antifungal voriconazole is first-line therapy for the treatment of invasive aspergillosis.[2] Before voriconazole became available in 2002, itraconazole was the azole of choice for treating aspergillosis and it is still used in countries where voriconazole availability is limited. Recently, a unique mechanism of resistance to the azole antifungals in *A. fumigatus* was identified.[3–6]

It was first identified in Europe from patients with *A. fumigatus* infections that were refractory to treatment; subsequently it was identified in isolates recovered from environmental sources in areas where agricultural azoles had been sprayed on crops.[3,7–9] The link between clinical resistance and agricultural azoles was strengthened when it was shown that agricultural azoles could confer cross-resistance to medical azoles in *A. fumigatus*.[10] Resistance has been subsequently identified in Asia, the Middle East, Africa, Australia and South America.[7,8,11–16] In the Netherlands, where the increase in resistance was first noted, azole resistance has risen to 5%–10% of all invasive aspergillosis cases and is as high as 30% in some high-risk wards.[17]

The unique resistance mechanism is an indel that consists of both a 34 bp tandem repeat in the promoter region of *CYP51A*, the azole target gene, and a substitution of leucine for histidine at codon 98 (denoted as TR_{34}/L98H). The combination of these two mutations confers cross-resistance to the clinical azole antifungals.[3] A second combination indel–substitution also specifically associated with isolates from the environment, TR_{46}/Y121F/T289A, was first detected in Europe and was soon identified in Asia and South America as well.[14,18,19]

An initial survey of 1026 *A. fumigatus* isolates collected from clinics throughout the USA from 2011 to 2013 did not identify any isolates with either the TR_{34}/L98H or the TR_{46}/Y121F/T289A mutation.[20] However, a report describing four patient isolates, two with TR_{34}/L98H and two with TR_{46}/Y121F/T289A, was published in 2015[21] followed by another report describing a third case of a patient isolate containing the TR_{46}/Y121F/T289A mutation.[22] In an effort to determine whether isolates containing TR_{34}/L98H or TR_{46}/Y121F/T289A could exist in the environment in the USA, we sampled crop debris from peanut fields with a history of exposure to the agricultural azoles tebuconazole and propiconazole, two of

the azoles that have been proposed to be the likely source of azole-resistant *A. fumigatus* in other countries[10,23] and both of which are actively used as agricultural fungicides in the USA.

Materials and methods

Environmental sampling, recovery, identification and susceptibility testing of A. fumigatus

Environmental sampling was carried out in experimental fields on University of Georgia experimental research farms (Tift County, GA, USA) in November 2015 after the crop harvest. A total of 34 specimens were collected from four separate peanut fields that had been sprayed with azole fungicides. Recent harvest crop debris and soils were collected from each of the four fields. In addition, the harvest debris from the previous years was collected from a single large compost pile, to which debris from individual fields had been deposited and composted for multiple years. Samples were taken from the interior of the pile. Isolation of *A. fumigatus* from crop debris specimens was accomplished by adding ~30 mL of a sterile 0.1 M solution of sodium pyrophosphate to the collection jar, which was vortexed vigorously for 30 s. The jar was allowed to sit for 1 min; 10 and 100 μL of the supernatant were each plated on two Sabouraud dextrose agar (SDA) plates supplemented with chloramphenicol (0.05 mg/L) and gentamicin (0.05 mg/L). Plates were incubated at 45°C for up to 7 days and colonies were harvested when they were 2–4 mm in diameter. The plated volume of supernatant was adjusted if necessary in a subsequent plating for each sample to achieve <10 total colonies per plate. The isolates were preliminarily identified by macroscopic and microscopic morphology and definitive identification was made by sequencing the tubulin gene as previously described.[24] Isolates were screened for resistance by plating on SDA containing 4 mg/L itraconazole. In addition some supernatants were plated directly onto SDA plates containing 4 mg/L voriconazole. Susceptibility testing of the isolates was performed by broth microdilution according to CLSI document M38-A2.[25]

Amplification of the CYP51A gene

Genomic DNA was extracted from mycelium as previously described.[13] The *CYP51A* gene and promoter were amplified using two primers and sequenced using eight additional primers as described previously.[20] The *CYP51A* sequence from *A. fumigatus* strain 237 (GenBank accession number AF338659) was used as the WT reference.

Microsatellite analysis

Isolates were genotyped by microsatellite analysis using the nine primer sets STRAF2a, STRAF2b, STRAF3a, STRAF3b, STRAF4a, STRAF4b, BDA, BDB and BDD as previously described.[26,27] eBURST analysis of microsatellite results was accomplished using eBURST V3 Imperial College London.[28]

Results

Out of the 34 environmental specimens that were collected, no *A. fumigatus* was isolated from 26 of them, including all of the recent harvest piles and soil from the individual fields. A total of 200 isolates of *A. fumigatus* were grown from eight specimens collected at different spots from a large compost pile containing peanut plant harvest debris from multiple fields that had accumulated over several years. Of these 200 isolates, 38 (19%) were able to grow on medium containing 4 mg/L itraconazole. Susceptibility testing confirmed the 38 isolates were resistant to itraconazole with MIC values of ≥16 mg/L and voriconazole MICs ranging from 0.5 to 4 mg/L. Sequencing of the *CYP51A* gene revealed 20 of the itraconazole-resistant isolates contained TR_{34}/L98H and none contained TR_{46}/Y121F/T289A.

To screen specifically for TR_{46}/Y121F/T289A, specimens from the large debris piles were plated directly onto SDA containing 4 mg/L voriconazole. Eighteen additional azole-resistant *A. fumigatus* isolates were identified. None of those isolates possessed TR_{46}/Y121F/T289A and only one possessed TR_{34}/L98H. However, four possessed at least one mutation known to be in the *CYP51A* genes of both susceptible and resistant isolates, such as F46Y/M172V/N248T/D255E/E427K, I242V and G254V (data not shown).

To determine the genetic relatedness of the isolates, microsatellite typing was performed on 51 of the isolates, 30 of which contained the TR_{34}/L98H mutation. Of these 30 isolates with the TR_{34}/L98H mutation, 22 (73%) were identical by microsatellite typing, suggesting they were clonal in origin. An additional six isolates differed from the clonal group by a single locus (a different locus for each of the six isolates), one more was different by two loci and one TR_{34}/L98H isolate had a completely unrelated microsatellite pattern (Figure 1). By contrast, the majority of the susceptible isolates had a unique microsatellite pattern, indicative of a complex population structure of *A. fumigatus*.

Discussion

This article demonstrates that TR_{34}/L98H azole-resistant *A. fumigatus* isolates can be found in an agricultural setting in the USA. This surveillance was not meant to be comprehensive or quantitative. Further strategic sampling in agricultural and non-agricultural areas should be conducted to assess the geographical boundaries of *A. fumigatus* resistance. It will also be important to better understand the structure of *A. fumigatus* natural populations in the USA to determine whether isolates with the TR_{34}/L98H mutation have been introduced from other sources or originated locally in the USA. A more comprehensive typing system like WGS may be able to distinguish between these two possibilities.[29] Although there have only been a few patients with disease caused by azole-resistant isolates with this mutation in the USA,[21] there needs to be heightened awareness among

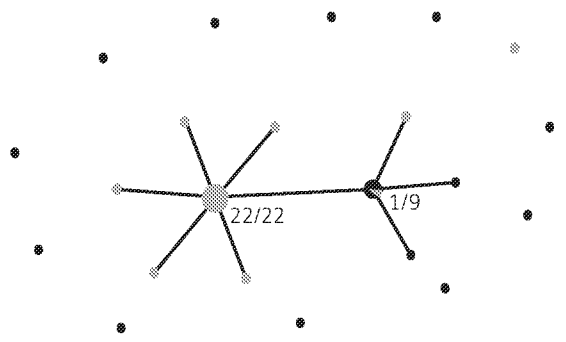

Figure 1. Genetic relationships among 51 isolates based on nine microsatellite loci visualized by eBURST. Each circle represents a distinct genotype; the size of a circle is proportional to the number of isolates within the genotype. Related genotypes are connected by lines; a line is equivalent to a one-locus difference. Genotypes with the TR_{34}/L98H mutation are light grey and azole-susceptible isolates are black. Numbers next to the circles indicate the number of isolates with the TR_{34}/L98H mutation out of the total number of isolates with the same genotype. If no number is placed next to the genotype it means that only a single isolate with this genotype has been recovered.

healthcare providers about possible resistance in patients with invasive aspergillosis. It is important to note that several studies have demonstrated that patients can be coinfected or co-colonized by both resistant and susceptible isolates at the same time, so culturing a susceptible isolate from a patient with a refractory infection does not rule out the presence of a resistant isolate.[30,31] While susceptibility testing of all isolates of *A. fumigatus* is not currently recommended in the USA by the IDSA,[2] it should be considered when patients have aspergillosis that is refractory to azole treatment.[32] Further surveillance and study for azole-resistant *A. fumigatus* is needed in both clinical and environmental areas in the USA.

Acknowledgements

We wish to acknowledge Shefali Jain and Grace Kim at the Centers for Disease Control for their laboratory assistance on this project, and Tim Brenneman of the University of Georgia for assistance at the surveillance site.

Funding

This study was supported by internal funding.

Transparency declaration

None to declare.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

References

1 Pappas PG, Alexander BD, Andes DR *et al.* Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin Infect Dis* 2010; **50**: 1101–11.

2 Patterson TF, Thompson GR 3rd, Denning DW *et al.* Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2016; **63**: e1–e60.

3 Mellado E, Garcia-Effron G, Alcazar-Fuoli L *et al.* A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob Agents Chemother* 2007; **51**: 1897–904.

4 Verweij PE, Mellado E, Melchers WJ. Multiple-triazole-resistant aspergillosis. *N Engl J Med* 2007; **356**: 1481–3.

5 Meis JF, Chowdhary A, Rhodes JL *et al.* Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. *Philos Trans R Soc B Biol Sci* 2016; **371**: 20150460.

6 Verweij PE, Chowdhary A, Melchers WJ *et al.* Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? *Clin Infect Dis* 2016; **62**: 362–8.

7 Snelders E, van der Lee HA, Kuijpers J *et al.* Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med* 2008; **5**: e219.

8 Snelders E, Huis In 't Veld RA, Rijs AJ *et al.* Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* 2009; **75**: 4053–7.

9 Verweij PE, Snelders E, Kema GH *et al.* Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect Dis* 2009; **9**: 789–95.

10 Snelders E, Camps SM, Karawajczyk A *et al.* Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One* 2012; **7**: e31801.

11 Chowdhary A, Kathuria S, Randhawa HS *et al.* Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the *cyp51A* gene in India. *J Antimicrob Chemother* 2012; **67**: 362–6.

12 Chowdhary A, Sharma C, van den Boom M *et al.* Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *J Antimicrob Chemother* 2014; **69**: 2979–83.

13 Lockhart SR, Frade JP, Etienne KA *et al.* Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *cyp51A* gene. *Antimicrob Agents Chemother* 2011; **55**: 4465–8.

14 Le Pape P, Lavergne RA, Morio F *et al.* Multiple fungicide-driven alterations in azole-resistant *Aspergillus fumigatus*, Colombia, 2015. *Emerg Infect Dis* 2016; **22**: 156–7.

15 Kidd SE, Goeman E, Meis JF *et al.* Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* 2015; **58**: 350–5.

16 Badali H, Vaezi A, Haghani I *et al.* Environmental study of azole-resistant *Aspergillus fumigatus* with TR_{34}/L98H mutations in the *cyp51A* gene in Iran. *Mycoses* 2013; **56**: 659–63.

17 Lestrade PP, Meis JF, Arends JP *et al.* Diagnosis and management of aspergillosis in the Netherlands: a national survey. *Mycoses* 2016; **59**: 101–7.

18 van der Linden JW, Camps SM, Kampinga GA *et al.* Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin Infect Dis* 2013; **57**: 513–20.

19 Chowdhary A, Sharma C, Kathuria S *et al.* Azole-resistant *Aspergillus fumigatus* with the environmental TR46/Y121F/T289A mutation in India. *J Antimicrob Chemother* 2014; **69**: 555–7.

20 Pham CD, Reiss E, Hagen F *et al.* Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011-2013. *Emerg Infect Dis* 2014; **20**: 1498–503.

21 Wiederhold NP, Garcia Gil V, Gutierrez F *et al.* First detection of TR34 L98H and TR46 Y121F T289A Cyp51 mutations in *Aspergillus fumigatus* isolates in the United States. *J Clin Microbiol* 2016; **54**: 168–71.

22 Vazquez JA, Manavathu EK. Molecular characterization of a voriconazole-resistant, posaconazole-susceptible *Aspergillus fumigatus* isolate in a lung transplant recipient in the United States. *Antimicrob Agents Chemother* 2015; **60**: 1129–33.

23 Chowdhary A, Kathuria S, Xu J *et al.* Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR_{34}/L98H mutations in the *cyp51A* gene in India. *PLoS One* 2012; **7**: e52871.

24 Balajee SA, Kano R, Baddley JW *et al.* Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. *J Clin Microbiol* 2009; **47**: 3138–41.

25 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi—Second Edition: Approved Standard M38-A2*. CLSI, Wayne, PA, USA, 2008.

26 de Valk HA, Meis JF, Curfs IM *et al.* Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J Clin Microbiol* 2005; **43**: 4112–20.

27 Bart-Delabesse E, Humbert JF, Delabesse E *et al.* Microsatellite markers for typing *Aspergillus fumigatus* isolates. *J Clin Microbiol* 1998; **36**: 2413–8.

28 Feil EJ, Li BC, Aanensen DM *et al.* eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004; **186**: 1518–30.

29 Abdolrasouli A, Rhodes J, Beale MA *et al.* Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *MBio* 2015; **6**: e00536.

30 Ahmad S, Joseph L, Hagen F *et al.* Concomitant occurrence of itraconazole-resistant and -susceptible strains of *Aspergillus fumigatus* in routine cultures. *J Antimicrob Chemother* 2015; **70**: 412–5.

31 Kolwijck E, van der Hoeven H, de Sevaux RG *et al.* Voriconazole-susceptible and voriconazole-resistant *Aspergillus fumigatus* coinfection. *Am J Respir Crit Care Med* 2016; **193**: 927–9.

32 Verweij PE, Ananda-Rajah M, Andes D *et al.* International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist Updates* 2015; **21-22**: 30–40.